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Transglutaminase Activity in Human and Rabbit Ear Comedogenesis: A Histochemical Study

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Using monodansylcadaverine as a fluorescent, lysine-substrate analogue, alterations in the histochemical profile of transglutaminase activity during human and coal tar-induced rabbit ear comedogenesis were examined. When compared to the normal pilosebaceous follicles of both species, a marked increase in enzyme-specific fluorescence in the hyperplastic epidermal lining and in the epithelium of the sebaceous duct was observed in human and rabbit early lesions. In mature human open and closed comedones, an intact band of transglutaminase activity corresponding to the differentiating epidermal cell layers was seen to surround the lesion. In mature rabbit comedones this band of activity was most striking. In most lesions, transglutaminase activity was closely apposed to the plasma membrane of the differentiating keratinocytes, the site of cornified envelope formation. In normal human and rabbit interfollicular epidermis the cornified layer immediately above the fluorescent granular layer was histochemically inactive, while the surface stratum corneum showed an increase in activity. A similar phenomenon was observed in many comedones. This pattern of activity may relate to the process of desquamation. Finally, the changes in transglutaminase activity observed during comedogenesis suggest the possibility that inhibitors of transglutaminase could be useful in acne therapy.

Whereas much acne research has been devoted to morphology and etiology, relatively little is known concerning the biochemistry of the abnormal epidermal differentiation which characterizes this disease. Transglutaminase (TG) activity is an important marker of normal interfollicular epidermal differentiation and in this study we have examined whether it shows an unusual pattern of expression in comedogenesis.

The primary function of TG activity in the epidermis appears to be a catalysis of ϵ-(γ-glutamyl)lysine cross-links of the protein contained in the cornified envelope [1]. To our knowledge the only study to date of TG activity in a cutaneous disease is that of Buxman and Wuepper [2] who used the fluorescent analogue, monodansylcadaverine (DC), and localized the activity of this enzyme in mammalian skin to the upper spinous layer and granular layer, with the latter site staining most intensely. Such histochemical staining was absent in psoriatic plaques even though active enzyme could be isolated from the same source [2]. In the study presented here we have examined changes in the histochemical activity of TG during human acne and in coal tar-induced rabbit ear comedogenesis.

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Abbreviations:
DC: monodansylcadaverine
IFE: interfollicular epidermis
TG: transglutaminase

MATERIALS AND METHODS

Human Biopsies

Male and female subjects in their 20's and 30's with noninflammatory acne of the back were selected for biopsy. They had not used topical or systemic acne medications for at least 1 month prior to biopsy and were otherwise healthy individuals. Healthy non-acne subjects were biopsied for controls. All subjects signed informed consent forms. Only open or closed comedones that were at the limit of detection with the unaided eye were selected for biopsy. In normal and some acne individuals, random sites on the back containing apparently lesion-free follicular orifices were biopsied. For biopsy, skin was locally anesthetized by injection of 1% lidocaine without epinephrine and a 3- or 4-mm hand punch was taken. Tissue was immediately frozen in O.C.T. compound (Lab-Tek Products, Naperville, Illinois) and stored at −70°C for no more than 1 month prior to cryostat sectioning. In all, 31 subjects were biopsied for a total of 13 closed comedones, 10 open comedones, 12 sebaceous follicles from normal individuals, and 11 sebaceous follicles from acne subjects.

Rabbit Ear Comedogenesis

Female New Zealand White rabbits weighing approximately 2 kg were purchased from Nitabell Rabbitry, Hayward, California, and housed under standard laboratory conditions. After 2 weeks of acclimation, rabbits were treated with a 0.25-ml application of 2% crude coal tar (Emerson Laboratory, Dallas, Texas) in Polylan (Amerchol Corp. Edison, New Jersey) 5 days a week for 2 weeks [3]. An automatic repeating pipette was used to dispense the coal tar to the inner ear canal of the right ear only. The left ear was untreated and served as a control. For histochemical determination of TG activity, animals were sacrificed at the appropriate times and 6-mm punch biopsies of the treated and control ear taken. Tissue was embedded and stored as described above for human samples.

Histochemical Assay

Tissue was serially cut in 8–10 μm-thick sections using a Cryostat II (Miles Laboratories, Inc., Naperville, Illinois) and air-dried on 0.1% poly-L-lysine-coated slides [4]. Poly-L-lysine does not interfere with TG histochemical activity (De Young and Ballaron, unpublished observation). DC in a modification of an existing technique [2] was used to localize TG activity. This technique identifies the location of the TG catalyzed binding of DC to the endogenous insoluble γ-glutamyl substrate. Sections were immersed for 30 min at 37°C in 0.05 M Tris-buffered saline (pH 8.1) containing 10 mM $CaCl_2$, 5 mM dithiothreitol, 2 mM DC, and 2.5% dimethylsulfoxide. The latter was used to solubulize the DC. We have found dimethylsulfoxide in concentrations as great as 10% to be without effect on the activity of partially purified epidermal TG (De Young, unpublished observations). Negative control sections were incubated as above with the exception that $CaCl_2$ and dithiothreitol were omitted and 10 mM EDTA added. After incubation, sections were washed at room temperature through 2 changes of buffer (2 min each), dehydrated through a graded series of alcohols to xylene, and coverslipped. The sections were observed with a Zeiss epifluorescence microscope equipped with a G365 exciter filter, FT 395 reflector, and a LP420 barrier filter (Carl Zeiss, Inc., New York, New York). Representative sections of each biopsy were stained with hematoxylin and eosin for light microscopic study.

RESULTS

Light Microscopy

Detailed histologic descriptions of human acne [5,6] and rabbit ear comedogenesis [7,8] have appeared elsewhere. Our

results are consistent with these and will not be discussed in detail here.

Human Interfollicular Epidermis (IFE)

As previously reported [2], TG activity is histochemically localized to the stratum spinosum and stratum granulosum of the epidermis. In addition, we have routinely observed that the horny layer immediately above the granulosum lacks activity while the portion of the horny layer in contact with the environment shows considerable activity (Fig 1). This activity is not observed in negative control sections and is therefore assumed to represent specific TG activity.

Human Sebaceous Follicle

In sebaceous follicles of both normal and acne subjects, TG activity runs the entire length of the infundibulum and is continuous with the band of activity seen in the IFE. It is confined to the differentiating epidermal cells of the infundibulum and sebaceous ducts feeding into the infundibulum. The sebaceous glands themselves are negative (Fig 2*A*). Often fluorescence at the mouth of the follicle is observed. Since this is sometimes associated with autofluorescent microbial colonies it is difficult to ascertain the contribution of specific TG activity. Fluorescence is, however, not observed in the horny cell layers immediately above the TG active layers of the infundibulum. Hair shafts when present are intensely autofluorescent (Fig 2*A*).

Human Early Comedones

We define early comedones as those lesions which histologically have a distended lumen, hyperplastic infundibulum, and, unlike mature comedones, retain a significant complement of intact sebaceous glands. We frequently observed such lesions in the tissue adjacent to mature lesions. In early comedones TG activity is found in the same loci as in the sebaceous follicle. However, fluorescence now defines a hyperplastic infundibulum and the plasma membrane of individual cells is easily discerned (Fig 2*B*). At the entrance of the sebaceous ducts into the lumen, intense fluorescence is observed and it also defines the plasma membrane of hyperplastic epithelial cells. This area appears to be fusing with the proximal infundibulum and thus the band of TG activity surrounding the lumen of the follicle appears more intact than observed in normal structures (Fig 2*B*).

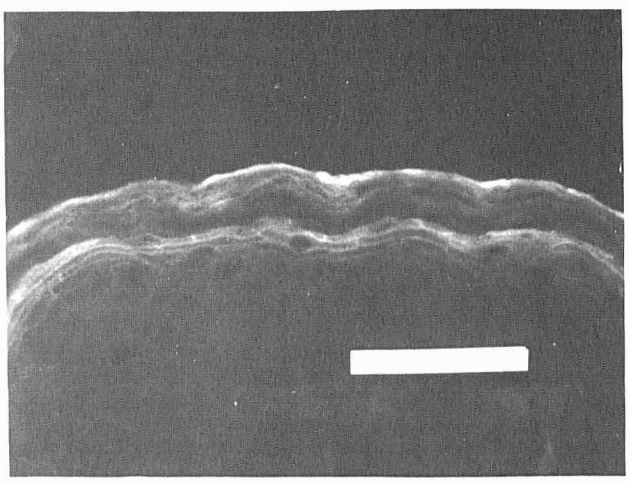


FIG 1. Normal human IFE, TG specific dansylcadaverine fluorescence. Bottom band of activity is localized to the upper layers of the living epidermis. The middle layer of stratum corneum is inactive, while the layer of stratum corneum at the surface shows activity. (For further explanation of this pattern of activity see Fig 5) *Scale bar* = 50 μm.

Human Open and Closed Comedones

Histologically these lesions have a distended lumen, an intact epidermal lining, that in many cases is thinner than that of early comedones, and a greatly diminished complement of sebaceous glands. Since these lesions differ significantly only in the size of their ostium, and since no differences in histochemical TG activity were seen, they are described together.

In the majority of serial sections of these lesions the band of

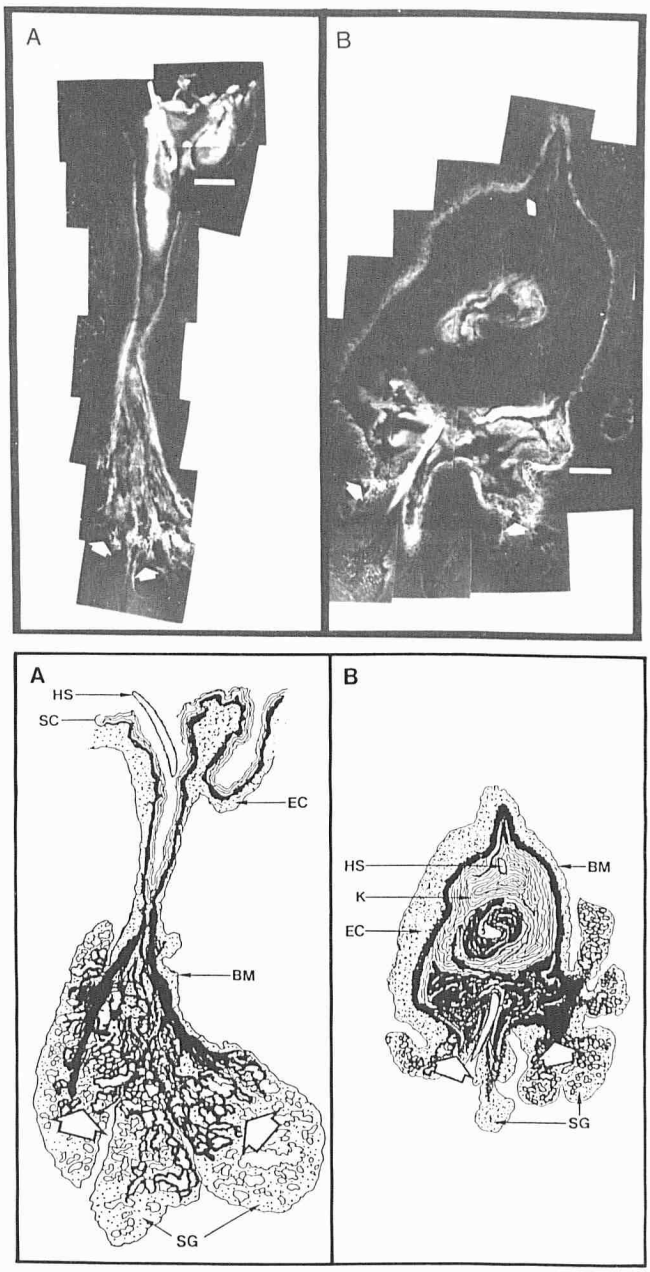
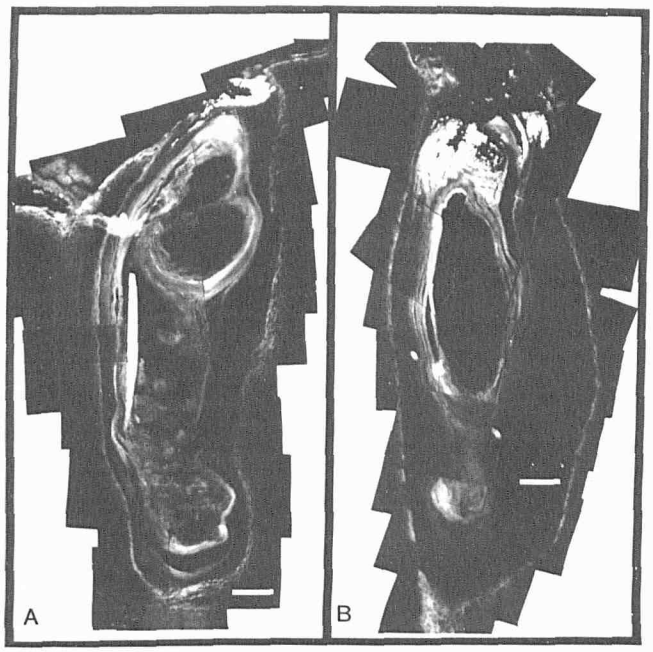


FIG 2. *Photomicrographs*: *A*, Normal human sebaceous follicle. TG activity in the infundibulum and sebaceous ducts. Fluorescence at follicular mouth is a combination of TG specific activity and autofluorescent bacterial colonies. Autofluorescent hair shaft is also visible. *Arrows* indicate insertion of sebaceous duct. *B*, Early lesion. Increased TG activity at insertion of sebaceous ducts (arrows). Band of activity surrounding lumen of follicle is pronounced. *Scale bars* = 100 μm. *Schematic diagrams*: These represent tracings of photomicrographs of H&E sections taken adjacent to the transglutaminase histochemical sections shown above. The location of TG activity is depicted by the heavy black areas. *BM* = epidermal basement membrane, *EC* = epidermal cells, *HS* = hair shaft, *K* = Keratin plug, *SC* = stratum corneum, *SG* = sebaceous gland.

TG activity completely surrounds the lesion except of course at the ostium (Fig 3). As was the case for normal skin, normal sebaceous follicles, and early lesions, fluorescence is not observed in the horny layers overlying the band of activity in the living epidermis. Again there is fluorescence in the central horny plug. In some cases this can be associated with autofluorescent microbes. However, in others, fluorescence cannot be observed in negative control sections, thus indicating specific TG activity. Many comedones contain yeast colonies at their mouths and these show a striking autofluorescence (Fig 3).

Normal IFE of the Inner Ear Canal of Rabbits

The pattern of TG activity in normal rabbit IFE (Fig 4*A*) is virtually identical to that of normal human IFE (see Fig 1); that is, specific fluorescence is observed in the upper differentiating layers of the living epidermis, disappears in the horny layers immediately above these, and reappears at the surface in contact with the environment (Fig 4*A*). We had observed a similar pattern of activity in human IFE (Fig 1). To determine



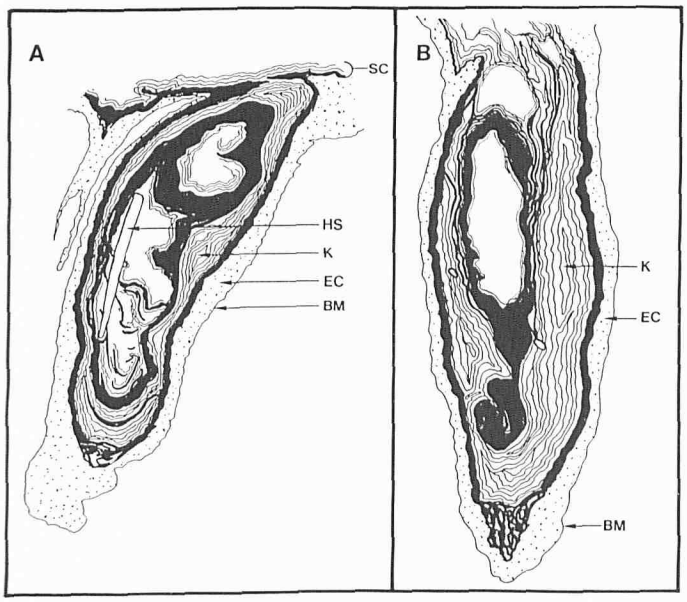
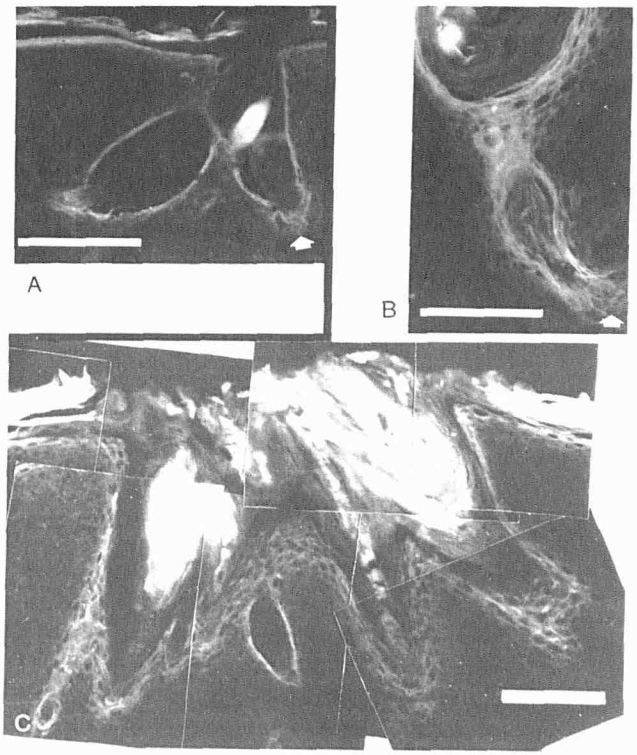


FIG 3. *Photomicrographs*: Human closed (*A*) and open (*B*) comedones. The central keratin plug of both is surrounded by an intact TG-positive epithelial lining. Activity apposed to the membranes of individual cells can be discerned. Fluorescence at the mouth of both follicles is due to yeast colonization while the fluorescence in the center of the follicles is TG specific. *Schematic drawings*: See legend to Fig 2.



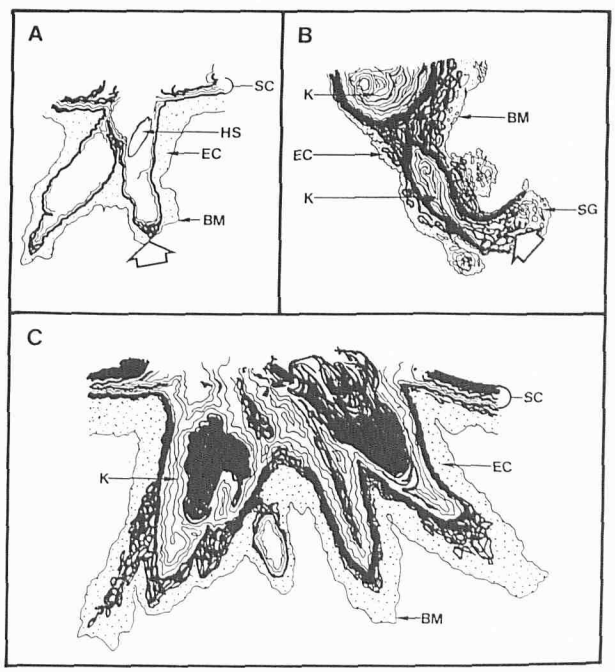


FIG 4. Rabbit ear comedogenesis. *Photomicrographs*: *A*, Normal pilosebaceous apparatus. TG activity is found in the differentiating epithelium lining the lumen and at the entrance into the lumen of the sebaceous ducts (*arrow*). *B*, Early lesion (after 5 coal tar treatments). Increased TG activity is seen in the hyperplastic, hypertrophic epithelia of the follicular lining and sebaceous duct. The outline of individual cells can be discerned. *C*, Mature comedone (after 10 coal tar treatments). TG specific fluorescence is dramatically apparent in the multilayered follicular lining, and inside the keratin plug. Scale bars = 100 μm. *Schematic drawings*: See legend to Fig 2.

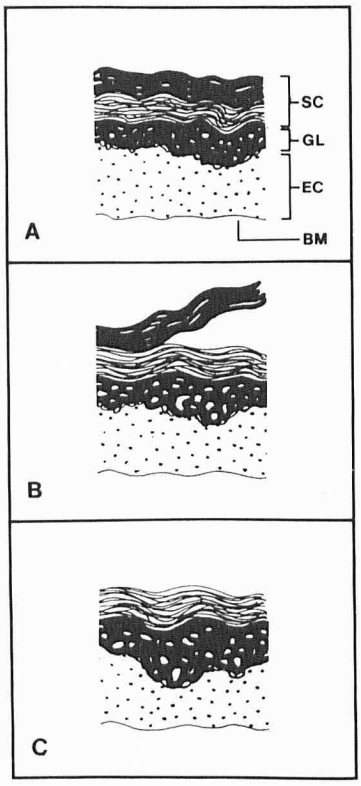


FIG 5. Schematic representation of the effect of tape-stripping normal rabbit inner ear canal skin. *A*, Distribution of TG activity in intact skin. Activity represented by the heavy black areas is found in the granular layer (*GL*) and in the surface stratum corneum (*SC*). The intermediate SC is negative. *EC* = epidermal cells. *BM* = basement membrane. *B*, After tape stripping 2 times the surface SC in some areas detaches from the intermediate SC. The TG activity remains with the detached SC. *C*, The surface has been totally removed by tape-stripping 5 times. The intermediate SC remains TG-negative.

whether this was due to a nonspecific edge effect we tape-stripped samples of rabbit ear skin prior to biopsy and TG assay. In some samples, tape-stripping caused the upper layers of the stratum corneum to separate from the lower layers, thereby creating an artificial new edge. In this situation the lower layers still lacked TG-specific fluorescence, indicating the observation is not due to an artificial edge effect (Fig 5).

Normal Rabbit Pilosebaceous Apparatus

As in normal human sebaceous follicles, TG activity in normal rabbit follicles is confined to a narrow band of differentiating epithelia lining the lumen and is continuous with the zone of activity in the IFE (Fig 4*A*). Also like the human, TG activity is seen in the sebaceous ducts entering the lumen. Fluorescence of the material within the lumen is usually not observed.

Changes in Rabbit Follicle During Coal Tar Treatment

When examined midway through the course of coal tar treatment (72 h after 5th treatment), profound changes in histochemical TG activity were observed (Fig 4*B*). The epithelial lining of the lumen and sebaceous ducts now show several layers of strikingly TG-active cells. This activity clearly outlines the cell membrane and is consistent with the tissue undergoing cornification (Fig 4*B*). This activity is not an artifact due to fluorescent constituents of the coal tar, since no fluorescence was observed in negative control sections.

Rabbit Ear Comedones

There is a particularly dramatic increase in the histochemical activity of TG in the epithelial lining of the rabbit ear comedo as seen 72 h after the 10th and last coal tar treatment (Fig 4*C*). Here a very hyperplastic, cornifying epithelium is evident, and TG activity is clearly seen outlining the plasma membranes of the spinous and granular cells. As in human lesions, fluorescence was absent or greatly diminished in the cornified layers above the granular cells and reappeared in the central horny plug. In the case of rabbits, however, this activity is always specific for TG since it was not observed in negative control sections and rabbit lesions do not support microbial growth.

DISCUSSION

In both human and rabbit comedogenesis, the principle changes in the expression of TG were the formation of an intact band of activity surrounding the follicular lumen and markedly increased expression of activity in sebaceous ducts. On the cellular level, increased TG activity was characterized by greatly enhanced fluorescence, outlining the inner aspect of the plasma membrane of differentiating cells. This is consistent with the site of action of epidermal TG which catalyzes the cross-linking of the epidermal cell envelope. Indeed, squames with thickened cornified envelopes appear de novo during comedogenesis when the keratinized follicular lining changes from disadherent strands to tightly packed layers [6].

One of the functions of the cornified envelope appears to be in providing the squamous cell with a relatively insoluble barrier that maintains the cell's integrity. The fact that these envelopes appear in the tightly adherent surface stratum corneum but not in the disadherent keratinized lining of normal sebaceous follicles may be coincidental or may indicate a role for cornified envelopes (and therefore TG) in cell-to-cell adhesion. For instance, the envelope could provide an anchor for intercellular attachments. The disruption of this envelope at the stratum corneum could then release the anchor with resultant sloughing of the superficial layers into the environment. Certainly, as discussed by Buxman and Wuepper [9], other biologic products of TG which are initially noted for their resistant structures, such as fibrin clots, rodent vaginal plugs, and the inner root sheath of hair follicles, are ultimately sloughed into the environment. An additional circumstantial relationship between cornified envelopes and the process of squamous cell adhesion is suggested by the action of the retinoids. These drugs which are effective anti-acne agents inhibit cornified envelope formation [10,11] and increase desquamation in vitro [12].

In this light it is provocative that we routinely observe a lack of TG-specific fluorescence in the squamous layer immediately above the TG-positive granular layer and a return of activity in the layer in contact with the external environment. Tape-stripping experiments in which only the surface stratum corneum was removed showed that this pattern of activity was not an artifactual edge effect. One possible explanation for this is that in the TG-negative layer all endogenous substrate is cross-linked and unavailable for acyl donation to exogenous DC, and at the surface in preparation for sloughing the endogenous cross-links are opened up and the substrate is again available to bind DC. An alternative but perhaps less likely explanation for the alternating bands of TG activity could be enzyme inactivation and reactivation.

To date, potent inhibitors of epidermal TG capable of suppressing cornified envelope formation in vivo have not been identified. The retinoids most probably inhibit cornified envelope production by decreasing envelope precursor levels [13]. In fact, De Young and Ballaron [14] have shown in vivo and Yuspa and his colleagues [11] have shown in vitro that retinoic acid actually increases levels of epidermal TG. Thus the effect of specifically inhibiting epidermal TG in intact normally differentiating skin or in acne skin, where we have now shown TG activity to be abnormally expressed, is presently unknown. The possibility that a specific inhibitor of epidermal TG could be efficacious in the treatment of acne is obviously intriguing and we feel this to be the central issue raised by this investigation.

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